

## SUPPLEMENTAL INFORMATION

### GH18 ENDO- $\beta$ -N-ACETYLGLUCOSAMINIDASES USE DISTINCT MECHANISMS TO PROCESS HYBRID-TYPE N-LINKED GLYCANS

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Running title: *N-glycan processing by endo- $\beta$ -N-acetylglucosaminidases*

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### 3. SUPPLEMENTAL REFERENCES

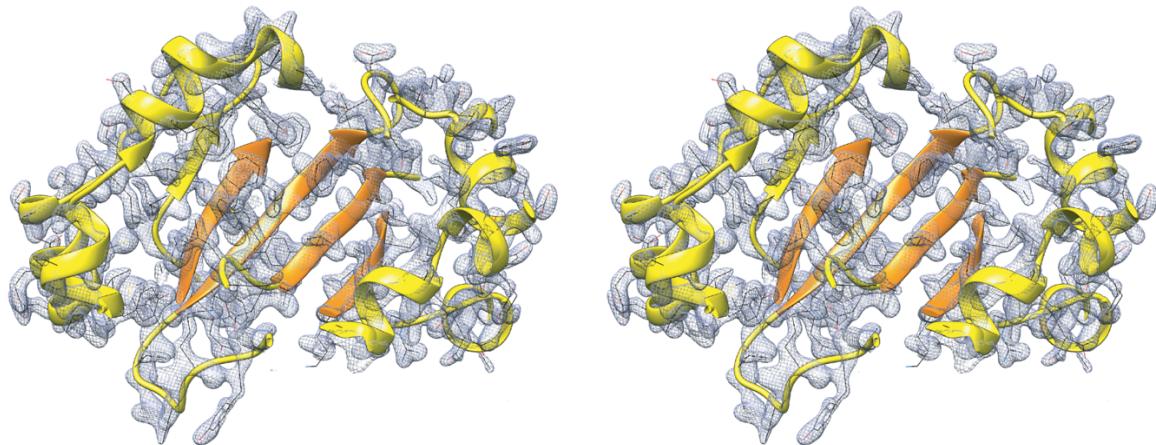
## 1. SUPPLEMENTAL TABLES

**Table S1. Data collection and refinement statistics.**

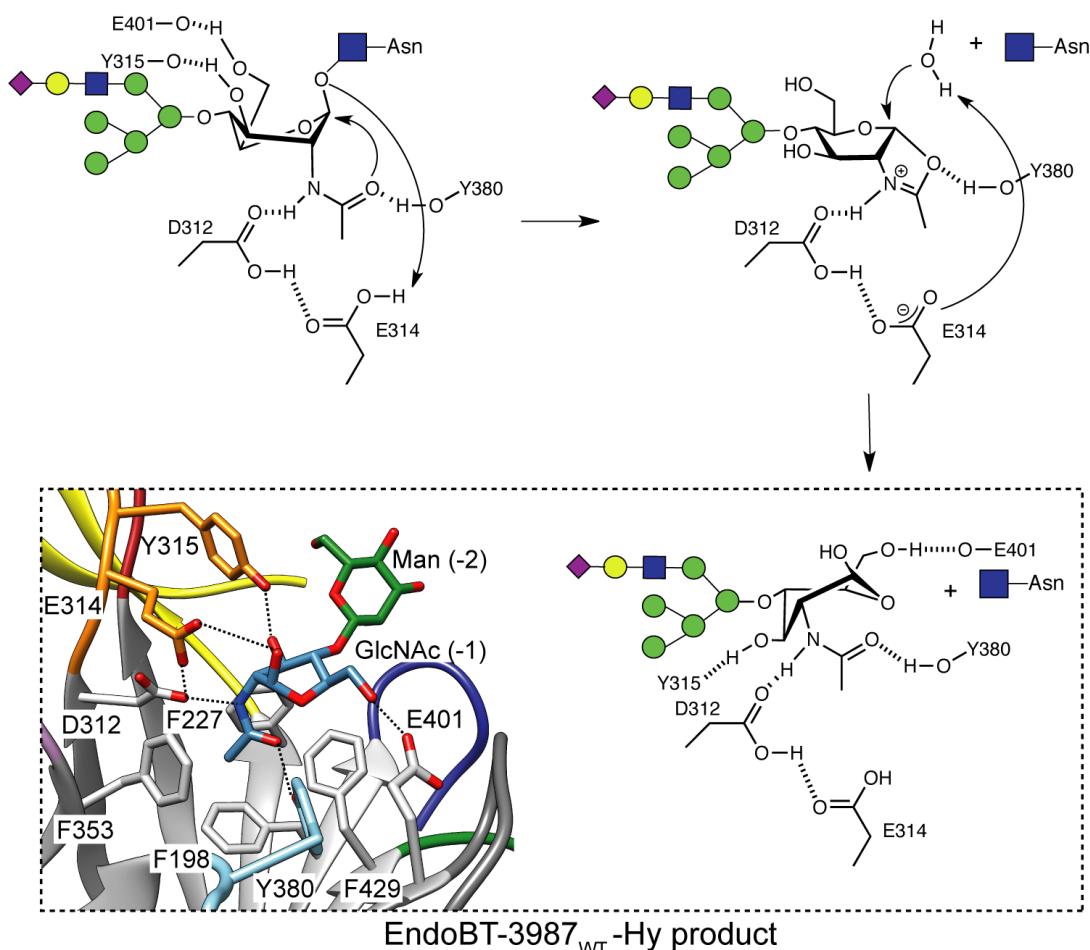
<b>EndoBT-3987<sub>WT-Hy</sub></b>	
PDB code	7NWF
Beamline	I24 (DLS) 6/04/2019
Wavelength (Å)	0.9792
Resolution range (Å)	45.9 - 2.0 (2.07 - 2.0)
Space group	P 21 21 21
Unit cell	49.29, 74.02, 125.54, 90, 90, 90
Total reflections	195095 (15200)
Unique reflections	30248 (2392)
Multiplicity	6.4 (6.4)
Completeness (%)	95.06 (76.87)
Mean I/sigma(I)	12.58 (2.57)
Wilson B-factor	24.51
R-merge	0.10 (0.63)
R-meas	0.11 (0.68)
CC1/2	0.99 (0.85)
CC*	0.99 (0.96)
Reflections used in refinement	30241 (2389)
Reflections used for R-free	1483 (114)
R-work	0.18 (0.23)
R-free	0.21 (0.27)
CC(work)	0.96 (0.89)
CC(free)	0.95 (0.77)
Number of non-hydrogen atoms	3711
macromolecules	3367
ligands	107
Protein residues	432
RMS(bonds)	0.013
RMS(angles)	1.23
Ramachandran favored (%)	97.44
Ramachandran allowed (%)	2.56
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.82
Clashscore	3.08
Average B-factor	25.65
macromolecules	25.35
ligands	32.24
solvent	26.84

Statistics for the highest-resolution shell are shown in parentheses

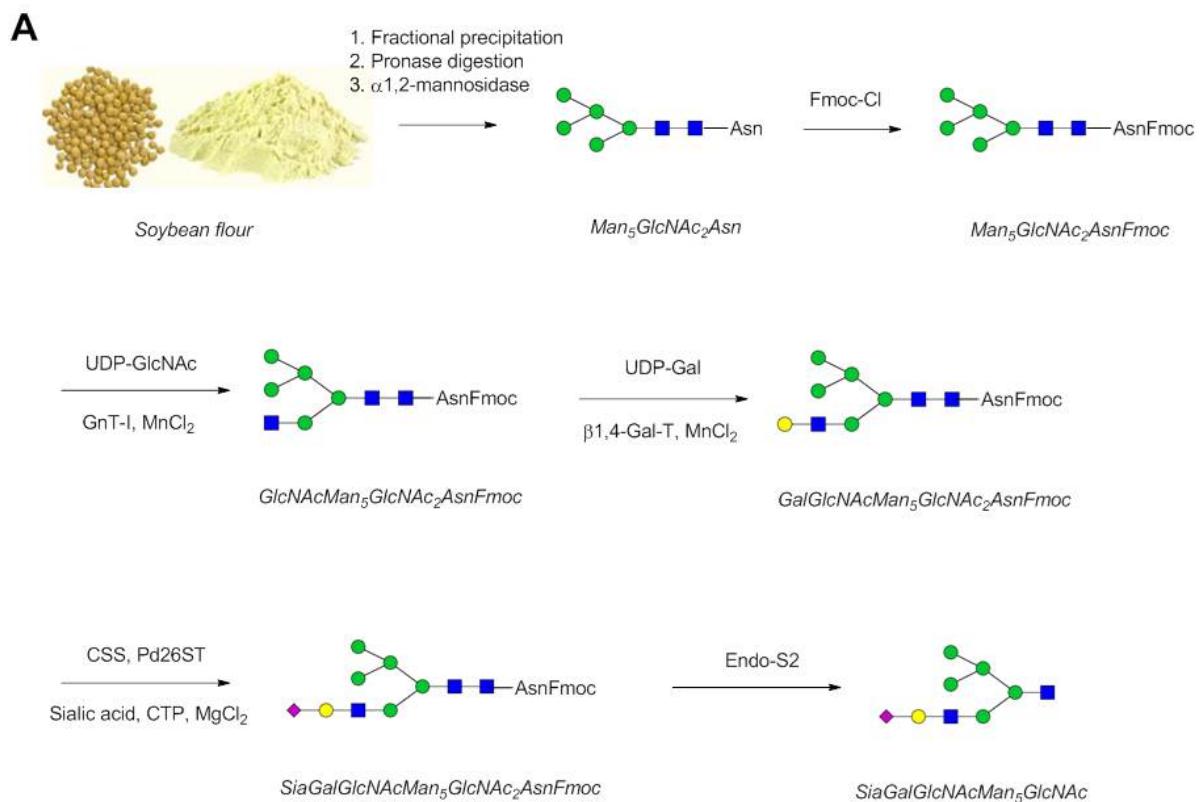
## 2. SUPPLEMENTAL FIGURES



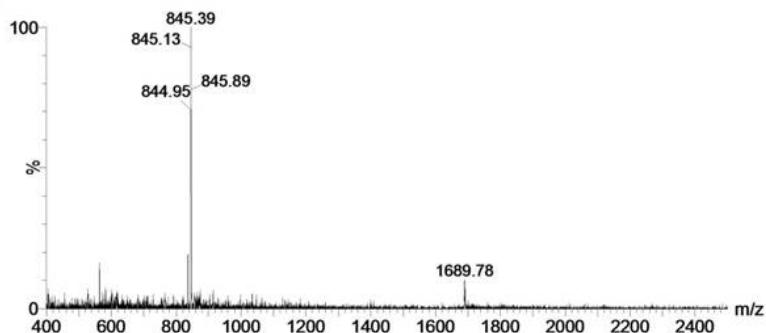
**Figure S1. Electron density maps of the refined EndoBT-3987 complex in the presence of GalGlcNAcMan<sub>5</sub>GlcNAc.** Stereo view of the final electron density maps (2mFo-DFc contoured at  $1\sigma$ ) corresponding to the EndoBT-3987in complex with the Hy-type *N*-glycan.



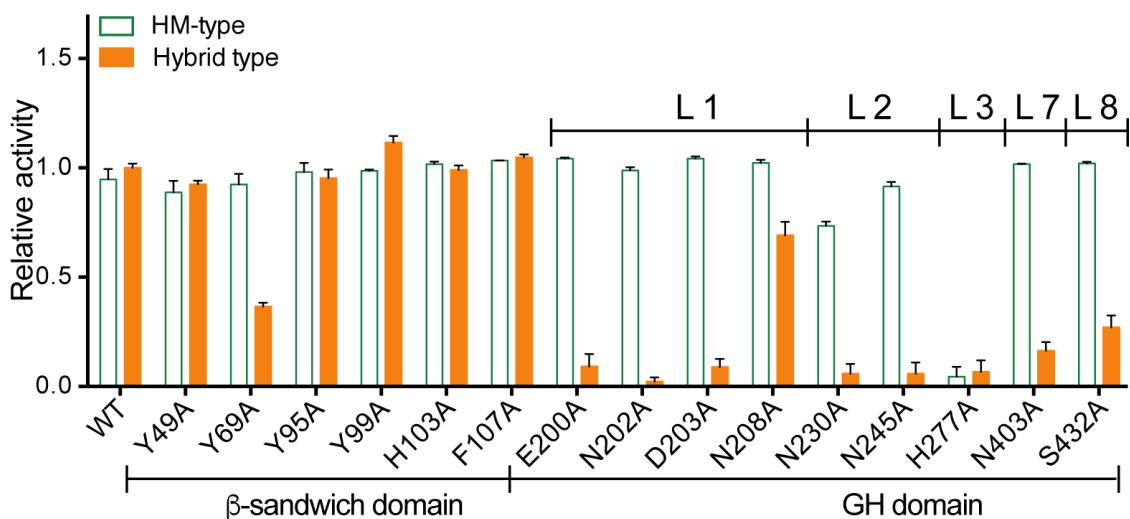
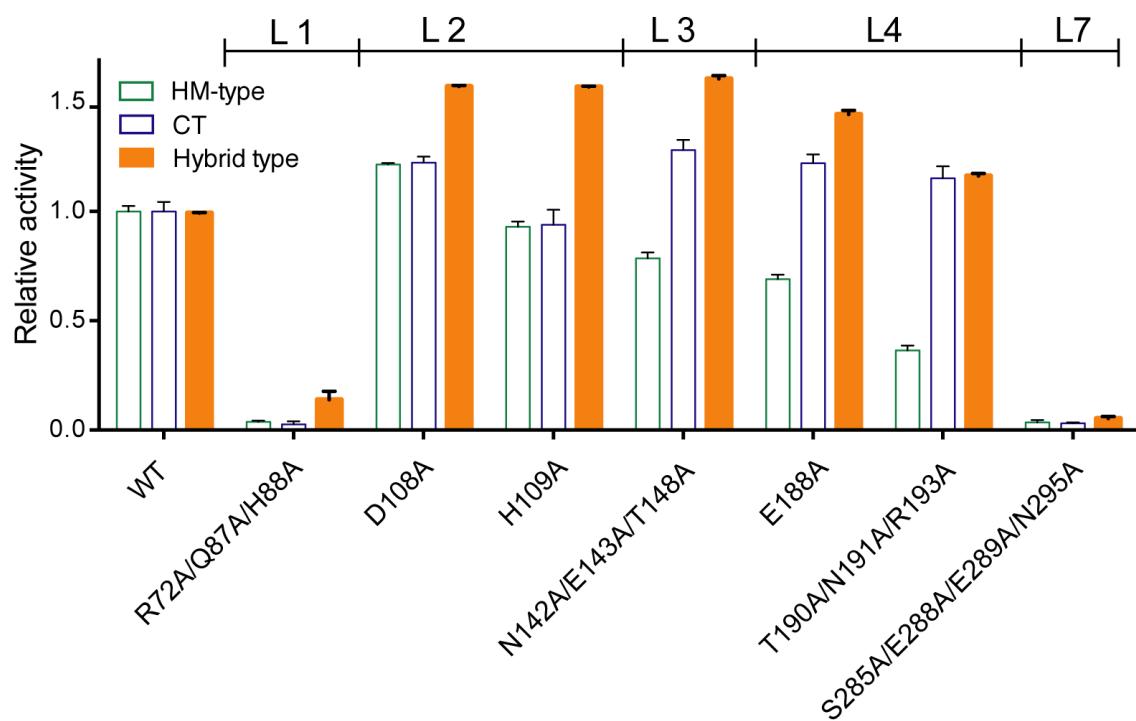
**Figure S2. The catalytic mechanism of EndoBT-3987.** In the first step, the *N*-linked Hy-type glycan substrate binds to the active site inducing the distortion of the GlcNAc (-1). In this step, the E314 residue protonates the glycosidic bond, acting as an acid, whereas the D312 residue orients the oxygen of the C2-acetamide group of GlcNAc (-1), which attacks the anomeric carbon of GlcNAc (-1) and leads to the formation of an oxazolinium intermediate. In the second step, E314 acts as a base, deprotonating a water molecule that performs a second nucleophilic attack and breaks the oxazoline ring, regenerating the hemiacetal sugar with retention of anomeric configuration.



**B**



**Figure S3. Biochemical synthesis of Neu5AcGalGlcNAcMan5GlcNAc.** (A) Chemoenzymatic synthesis of Hy-type N-glycan. (B) ESI-MS spectrum of the synthetic Hy-type N-glycan.

**A****EndoBT-3987 mutants****B****EndoS2 mutants**

**Figure S4. Comparison of hydrolytic activity of EndoBT-3987 and EndoS2 against Rituximab with defined glycoforms.** (A) Hydrolytic activity of EndoBT-3987 and mutants determined by LC-MS analysis against HM-Rituximab (white and green bars) previously described<sup>1</sup> and Hy-Rituximab (orange bars) performed in this study. (B) Hydrolytic activity of EndoS2 and mutants determined by LC-MS analysis against HM-Rituximab (white and green bars) and Rituximab (white and blue bars) previously described<sup>2</sup> and Hy-Rituximab (orange bars) performed in this study. The hydrolytic activity data is normalized against the activity of EndoBT-3987 and EndoS2 wild type against each Rituximab substrate.

### **3. SUPPLEMENTAL REFERENCES**

- (1) Trastoy, B.; Du, J. J.; Klontz, E. H.; Li, C.; Cifuentes, J. O.; Wang, L. X.; Sundberg, E. J.; Guerin, M. E. Structural Basis of Mammalian High-Mannose N-Glycan Processing by Human Gut *Bacteroides*. *Nat. Commun.* **2020**, *11* (1), 899.
- (2) Klontz, E. H.; Trastoy, B.; Deredge, D.; Fields, J. K.; Li, C.; Orwenyo, J.; Marina, A.; Beadenkopf, R.; Günther, S.; Flores, J.; Wintrode, P. L.; Wang, L. X.; Guerin, M. E.; Sundberg, E. J. Molecular Basis of Broad Spectrum N-Glycan Specificity and Processing of Therapeutic IgG Monoclonal Antibodies by Endoglycosidase S2. *ACS Cent. Sci.* **2019**, *5* (3), 524–538.